Version 08.31.2012

R-PE Antibody All-In-One Conjugation Kit

Technical Manual

Catalog # A-9001-006

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Safety Information

WARNING – CHEMICAL HAZARD. Some chemicals used can be potentially hazardous, and can cause injury or illness.

- Read and understand the Material Safety Data Sheets (MSDS) available at Solulink.com before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (e.g. safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's clean-up procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling and disposal.

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Chapter 1: Introduction

A. User Manual

This manual provides instructions for using the R-PE Antibody All-in-One™ Conjugation Kit.

This chapter contains the following sections:

Purpose of Manual

Intended Users

Customer Service and Technical Support

B. Purpose of Manual

Each R-PE Antibody All-in-One™ Conjugation Kit provides all the necessary reagents and components to produce two (2) R-PE antibody conjugates in about 6 hours.

Use of the kit (for each antibody) results in:

- The modification of a user-supplied antibody (100 µg) with S-HyNic.
- The conjugation of a HyNic-modified antibody with 4FB-PE, resulting in the formation of an R-PE Antibody conjugate.
- The isolation of highly purified R-PE Antibody conjugates using a dual purification process (magnetic affinity beads and Q spin filter)

C. Intended Users

The R-PE Antibody All-in-One™ Kit is designed for users with minimal or no conjugation experience allowing them to prepare customized, high purity, ready-to-use R-PE Antibody conjugates in a single day.

D. Customer Service and Technical Support

Additional technical information can be found at:

Telephone

1-888-625-0670 (Toll Free)

Fax

1-858-625-0770

Email

Solulink@Solulink.com

Address

Solulink-The Conjugation Company

9853 Pacific Heights Blvd, Ste H

San Diego, CA 92121

Chapter 2: Overview of Conjugation

A. Product Description

Each R-PE Antibody All-in-One™ Conjugation kit is designed to produce two high quality ready-to-use R-PE antibody conjugates. Each conjugation requires 100 µg of user supplied mammalian IgG from any species. The kit is designed to produce heterodimer conjugates (1:1 R-PE to IgG ratio) devoid of virtually all free R-PE or residual IgG. Any suitably purified monoclonal or polyclonal antibody free of protein carriers or stabilizers (e.g. BSA or gelatin) can be used to form the conjugate. The kit employs a dual purification strategy using magnetic affinity beads and a Q spin filter. Conjugates produced are fully compatible with all demanding downstream applications including flow-cytometry and/or sensitive microarray detection. Each kit contains sufficient reagents to produce two ready-to-use R-PE antibody conjugates (~125 µg each).

B. All-in-One™ Conjugation Technology

Conjugation Chemistry

HydraLinK™ chemistry is based on the use of two complementary heterobifunctional linkers; **S-HyNic** and **Sulfo-S-4FB** (Figure 1). **S-HyNic** (Succinimidyl-6-hydrazino-nicotinamide) is first used to modify and incorporate protected aromatic hydrazines (HyNic groups) into the antibody via acylation of lysine residues. In a similar fashion the second linker, **Sulfo-S-4FB** (Sulfo-N-succinimidyl-4-formylbenzamide) is used by Solulink to form a pre-activated form of R-PE called 4FB-PE (provided in the kit). Incubation of the HyNic-modified antibody with pre-activated 4FB-PE in the presence of aniline as catalyst leads to rapid and efficient conversion of the antibody to conjugate through formation of stable bis-arylhydrazone bonds (Figure 2).

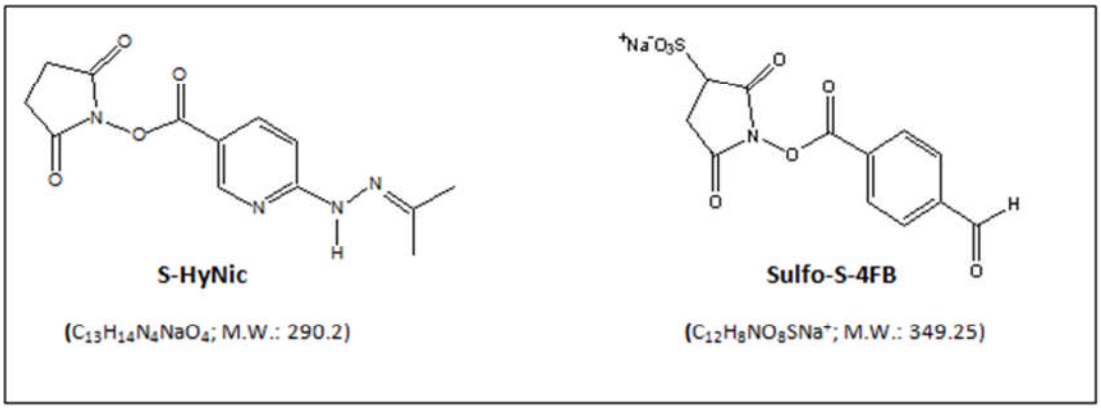


Figure 1. Structure of S-HyNic and Sulfo-S-4FB linkers used to conjugate R-PE to antibody.

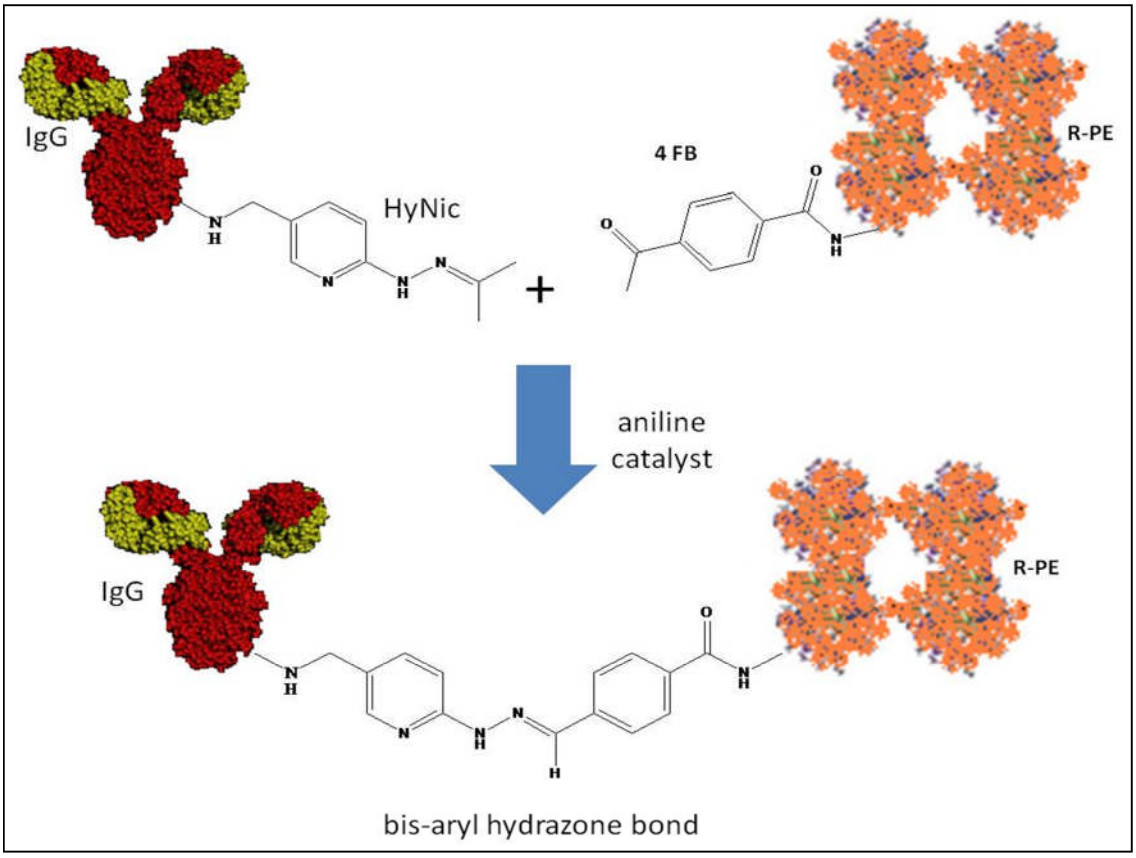


Figure 2. Conjugation chemistry used in linking IgG to R-PE.

Purification Technology

This All-in-One™ kit uses a two-stage purification process to generate highly purified R-PE/antibody conjugates as illustrated in Figure 3. Kits have been engineered to consistently produce a 1:1 heterodimer product starting with any mammalian antibody. The perfect heterodimer ratio (1:1) is made possible by the recent discovery that hydrazone bond formation is rapidly and efficiently catalyzed in the presence of aniline[1,2,3]. Aniline facilitates hydrazone bond formation between biomolecules at low protein concentrations and equivalent mole-ratios, both of which are necessary for the consistent formation of a heterodimer. In the first stage of the purification process the 1:1 heterodimer and any excess un-conjugated IgG are purified away from excess 4FB-PE using a magnetic affinity matrix or bead. In a second purification stage, a novel Q spin filter selectively binds conjugate but excludes free IgG from binding based on well-known biophysical properties of nearly all antibodies[4,5]. This novel dual purification process completely avoids costly and time consuming column chromatography procedures while removing all traces of free IgG and virtually all free R-PE. The result is a high activity conjugate consisting primarily of 1:1 heterodimer (>80%) with minor residual quantities of 1:2 heterodimer (~20%) in

high yield (see Appendix). All purified conjugates are ready for the most demanding downstream applications

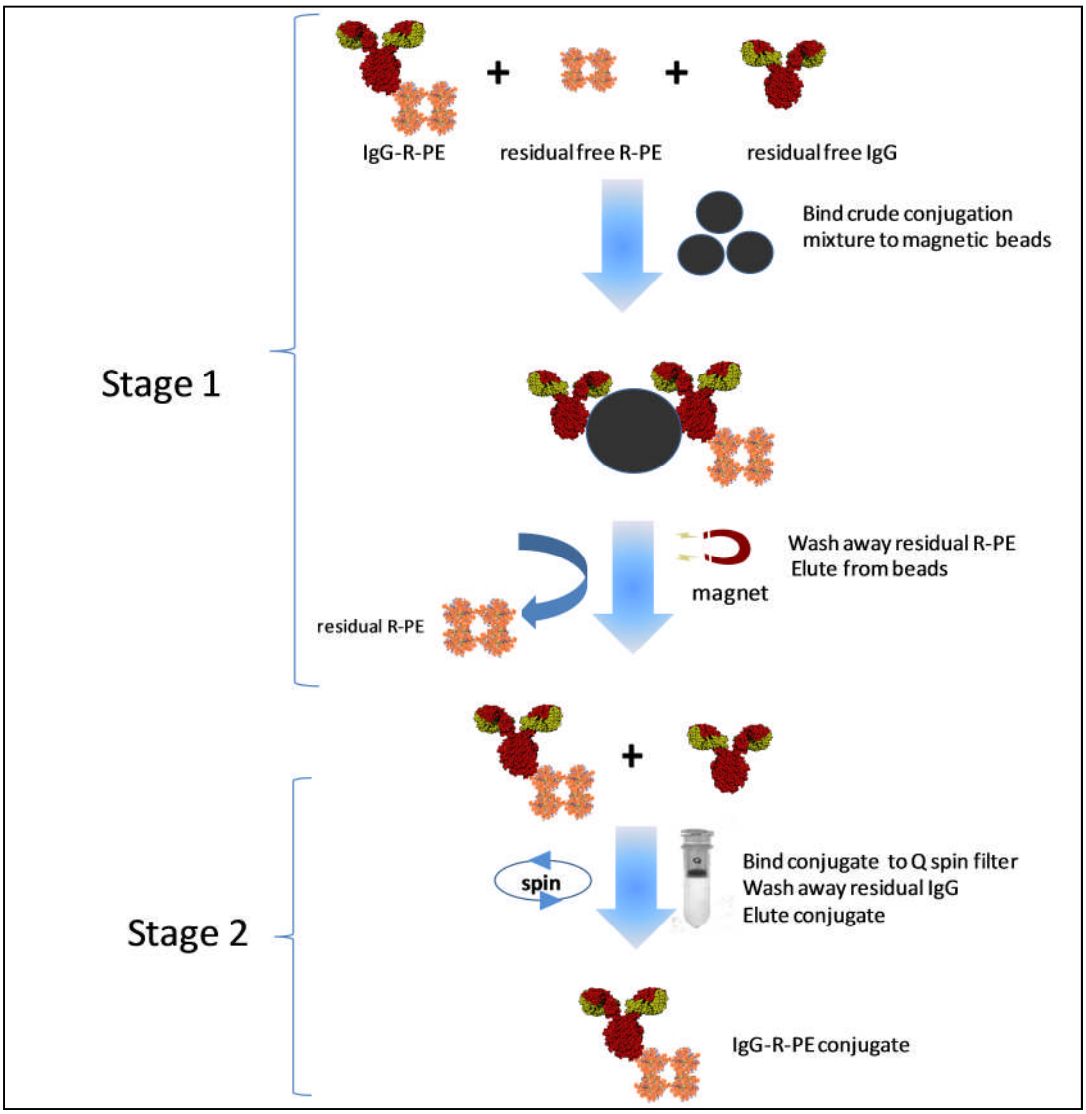


Figure 3. Two-stage purification of IgG-R-PE conjugate.

C. All-in-One™ Conjugation Process Summary

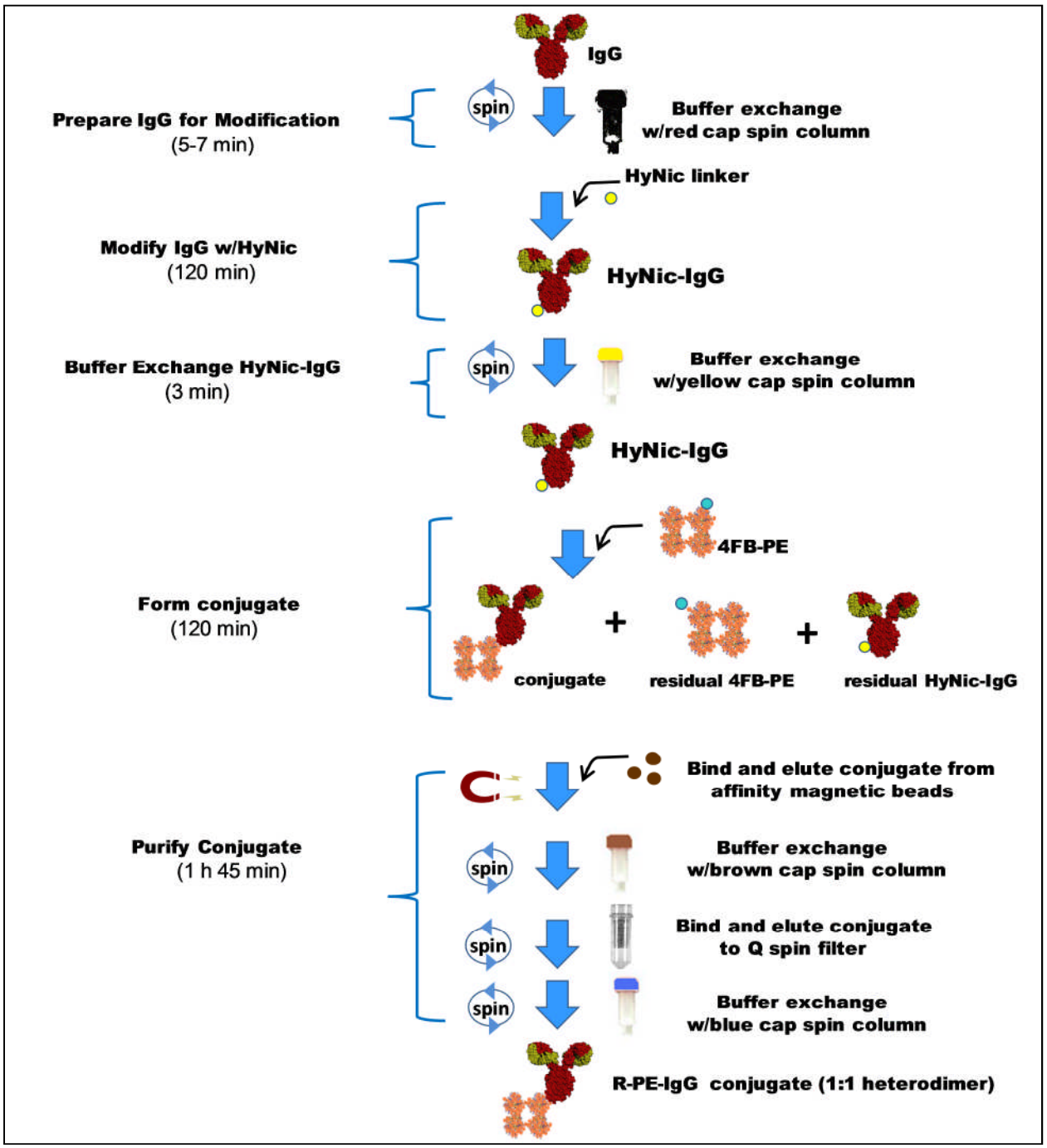


Figure 4. All-in-One™ conjugation process summary.

D. Materials Provided and Storage Conditions

Components	Amount	Storage conditions
S-HyNic	2 x 100 µg	Keep desiccated at or below room temperature within desiccated foil pouch
4FB – R-PE	2 x 48 µl	Keep refrigerated (2-8°C)
Buffer A	5 mL	Keep refrigerated (2-8°C)
Buffer B	5 mL	Keep refrigerated (2-8°C)
Buffer C	5 mL	Keep refrigerated (2-8°C)
Buffer D	0.5 mL	Keep refrigerated (2-8°C)
Buffer E	5 mL	Keep refrigerated (2-8°C)
Buffer F	0.5 mL	Keep refrigerated (2-8°C)
Anhydrous DMF	500 µL	Keep refrigerated (2-8°C)
Affinity Magnetic Beads	2 x 100 µL	Keep refrigerated (2-8°C)
Red Cap Spin Column	2	Keep refrigerated (2-8°C)
Yellow Cap Spin Column	2	Keep refrigerated (2-8°C)
Brown Cap Spin Column	2	Keep refrigerated (2-8°C)
Blue Cap Spin Column	2	Keep refrigerated (2-8°C)
Q Spin Column & Q Collection Tubes	2/4	Room temperature or refrigerated (2-8°C)
Collection Tubes	16	Room temperature

E. Additional Materials Required But Not Provided

Bradford Protein Assay Reagents (verification of final conjugate concentration)
Standard UV-VIS or NanoDrop™ Spectrophotometer
Pipettes (P-10, P-100, P-1000) and tips
Microcentrifuge (e.g. Eppendorf or MicroMax)
1.5 ml microfuge tubes

Chapter 3: All-in-One™ Conjugation Protocol

Note - Before use, remove the kit from the refrigerator and allow all kit components to warm up to room temperature for at least 30 minutes.

A. IgG Sample Preparation (5 minutes)

Antibodies come in two physical forms, solids or liquids. Individual samples can vary significantly in the amount of packaged IgG (protein mass) and/or concentration (mg/ml). We highly recommend that IgG concentrations be confirmed either by Bradford protein assay or A280 whenever possible. The All-in-One™ conjugation protocol requires antibody samples to be free of protein carriers such as BSA or gelatin before proceeding. A 100 µg mass of antibody is required to start the procedure. Depending on the state of your initial sample (solid or liquid), proceed as follows:

Antibody is in Solid Form (e.g. lyophilized powder)

Resuspend the lyophilized antibody (100 µg free of protein additives gelatin or BSA) in 100 µl **Buffer A** to obtain a 1 mg/mL solution. If the antibody sample contains less than 100 µg per vial (e.g. 50 µg), resuspend the antibody in the requisite number of vials equivalent to a 100 µg in 100 µl **Buffer A** to obtain a 1 mg/mL solution.

Proceed to step b.

Antibody is in Liquid Form (e.g. PBS or TBS Buffer)

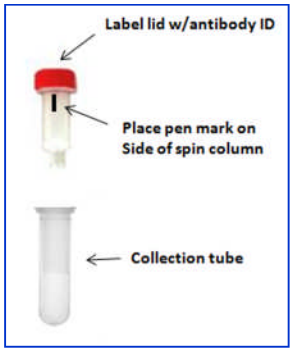
If the antibody sample is in liquid form at 1 mg/ml, simply transfer 100 µl to a labeled microfuge tube (100 µg). If the sample is in liquid form at a concentration greater than 1 mg/ml, transfer a volume equivalent to 100 µg antibody to a labeled microfuge tube and add **Buffer A** to obtain a 1 mg/ml solution. If a sample is at a concentration less than 1 mg/ml, concentrate the sample to 100 µL at 1 mg/mL using any suitable ultra-filtration spin filter (e.g. Amicon or VivaSpin 500) as described in the Appendix. A concentration filter is not provided with this kit.

Proceed to step b.

B. Buffer Exchange IgG (3 minutes)

1. Prepare a spin column (red cap) by twisting off the bottom closure and loosening the red cap (do not remove). Place the spin column into a collection tube (provided).

2. Mark the top of the red cap using an indelible pen to identify the sample and place a vertical mark on the side of each spin column as shown next.



3. Place the assembly into the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor.
4. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from the collection tube. The column matrix will appear white in color. Place the column back into a new, empty collection tube (provided).
5. Remove the red cap; load the antibody sample (100 µL at 1 mg/mL) to the top of the dry resin bed; loosely recap and place the column back into the collection tube.
6. Orient the spin column mark outward as before and centrifuge at **1,500 x g for 2 minutes**. Use a balance tube opposite the assembly.

> Note - Rotor speed should be set to 1500 x g (RCF) and not 1500 x rpm (RPM). The volume recovered should always be approximately the same volume loaded on the spin column (e.g. 100 ± 10 µL). If the recovered volume is low, the centrifuge may require recalibration. It is recommended to re-centrifuge at the appropriate speed in an attempt to recover the full volume (i.e. 100 µL).

7. Transfer the solution from the bottom of the collection tube to a 1.5 mL tube and label appropriately.

C. HyNic Modification of IgG (2 h)

1. Add 20 µl DMF to S-HyNic reagent vial. Pipette the solution up and down to resuspend the reagent pellet.

   Note-before DMF addition there is a small but visible pellet at the bottom of the vial.

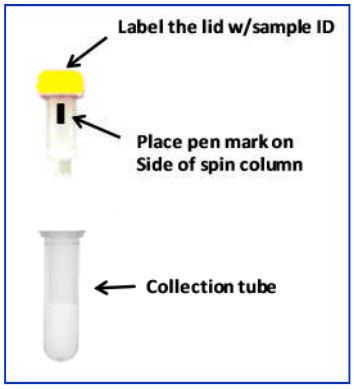
2. Add 1.5 µl dissolved S-HyNic reagent to the antibody solution (100 µl @ 1mg/mL) prepared in the previous section. Pipette the solution up and down to mix.

3. Incubate the reaction for 2 h at room temperature.

D. Buffer Exchange IgG (3 minutes)

1. Five minutes before the end of the HyNic modification reaction, prepare a spin column (yellow cap) by twisting off the bottom closure and loosening the yellow cap (do not remove). Place the spin column into a collection tube (provided).

2. Mark the top of the yellow cap using an indelible pen to identify the sample. Also place a vertical mark on the side of each spin column as shown on the next page.



3. Place the assembly into the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor. Use an appropriate balance tube opposite the assembly.

4. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from the collection tube. The column matrix will appear white in color. Place the column back into a new, empty collection tube (provided).
5. Open the yellow cap; load the now completed antibody/HyNic modification reaction (~ 100 µL) to the top of the dry resin bed; loosely cap and place the column back into the collection tube.
6. Orient the spin column mark outward as before and centrifuge at **1,500 x g for 2 minutes.** Use a balance tube opposite the spin filter.
7. After centrifugation, transfer the solution from the bottom of the collection tube to a 1.5 mL tube. Label the sample appropriately (e.g. HyNic-IgG).

E. Conjugate Formation (2 h)

1. Spin the dark brown vial containing 4FB-modified R-PE (5 seconds @ 1000 x g) to collect the contents at the bottom of the tube.
2. Transfer 48 µl 4FB-modified R-PE to the tube containing HyNic-modified antibody; pipette up and down to mix.
3. Incubate in the dark for 2 h at room temperature.

F. Conjugate Purification Stage 1: Affinity Isolation (1 ¾ hours)

Magnetic Affinity Bead Prep

1. Briefly centrifuge 100 µL affinity magnetic beads (black slurry) at **1000 x g for 5 seconds** to collect the bead contents at the bottom of each tube. Place the beads on a magnet for 10 seconds to pellet. Remove and discard the supernatant.
2. Add 400 µL **Buffer B** to the bead slurry and gently vortex to mix.
3. Before the beads resettle, place the beads on the magnet for 10 seconds to pellet; then carefully remove and discard the supernatant without disturbing the pellet.

4. Wash the beads by repeating steps 2 and 3 **two (2) additional** times. After discarding the final wash, immediately proceed to the next step.

Bind Conjugate to Affinity Beads

1. Away from the magnet, add the completed conjugation reaction (~ 150 µL from step E.3) to the washed bead pellet. Gently mix the slurry up and down 3-4 times using a P-1000 pipette. **Never vortex the beads after the conjugate mixture is added.**

   Tip: set the volume on the P-1000 pipetman on 100 µL before pipette mixing.

2. Let the slurry settle and incubate for **10 minutes** away from the magnet.
3. Gently pipette the bead slurry up and down with a P-1000 pipette 3-4 times to remix and incubate for another **10 minutes** away from the magnet.
4. Repeat step 3 **one (1) additional time**; total incubation time 30 minutes away from the magnet.
5. Away from the magnet, gently pipette the settled slurry up and down to mix and immediately place the tube on the magnet before the beads resettle. Once the beads pellet to the side (10-20 seconds) discard the supernatant without disturbing the pellet.

Wash Magnetic Beads

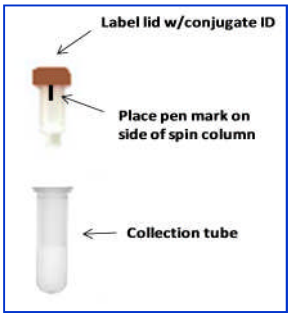
1. Add 400 µL **Buffer C** to the bead pellet and gently pipette (P-1000) the slurry up and down several times to wash. Allow the beads to resettle for 3 minutes on the bench top.
2. Once again pipette the slurry up and down and immediately place on a magnet before the beads resettle. Remove and discard the supernatant without disrupting the pellet.
3. Repeat step 1-2 **three (3) additional times**; always discard the supernatant between washes.

Elute Conjugate from Beads

1. Remove the beads away from the magnet and elute the conjugate by adding 50 µL **Buffer D** to the pellet**.**
2. Gently and slowly mix the bead slurry up and down with the aid of a pipetman.
3. Allow the beads to incubate away from the magnet for **4 minutes**.
4. Repeat step 2 and 3 **two (2) additional times**; total incubation time - **12 min.**
5. Gently resuspend the settled slurry by pipetting up and down and immediately place the beads on the magnet before they resettle.
6. Transfer the highly colored supernatant (50 µL) containing the eluted conjugate to a new 1.5 mL tube and label appropriately.
7. Continue elution of the conjugate by repeating steps 1 through 6 **two (2) additional times**; pooling all conjugate fractions into the same tube (150 µL final volume).

G. Buffer Exchange Conjugate (3 minutes)

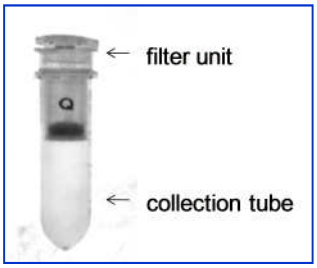
1. Prepare a spin column (brown cap) by twisting off the bottom closure and loosening the brown cap (do not remove). Place the spin column into a collection tube (provided).
2. Mark the top of the brown cap using an indelible pen to identify the sample. Also place a vertical mark on the side of each spin column as shown below.



3. Place the assembly into the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor. Use a balance tube opposite the spin filter
4. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from the collection tube. The column matrix will appear white in color. Place the column back into a new, empty collection tube (provided).
5. Open the brown cap; load 150 µL eluted conjugate to the top of the dry resin bed; loosely cap and place the column back into the collection tube.
6. Orient the spin column mark outward as before and centrifuge at **1,500 x g for 2 minutes**. Use an appropriate balance tube opposite the spin filter.
7. After centrifugation, add 300 µL **Buffer E** to 150 µL of conjugate at the bottom of the collection tube and pipette up and down to mix (450 µL total). Set aside on the bench.

H. Conjugate Purification Stage 2: Q Spin Filter (35 min)

1. Pre-wet a Q spin filter by adding 200 µL **Buffer E** to the top of the unit (see below) and incubate for 2 minutes.

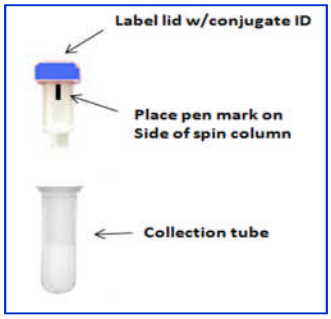


2. Place the assembly in the centrifuge and orient the letter Q towards the center of the rotor; spin at **2,000 x g for 4 minutes**; discard the flow-through from collection tube and place the filter back into the empty collection tube.
3. Load the R-PE Antibody conjugate (450 µL) from the previous section to the top of the filter unit and allow to incubate for 2 minutes with the filter.

4. Place the oriented assembly in the centrifuge and spin at **2,000 x g for 4 minutes;** discard the flow-through from the collection tube and place the filter back into the bottom empty collection tube. **Note-** a highly pink color will appear bound to the top of the Q filter membrane.
5. Add 400 µL **Buffer E** to the filter unit; orient in the centrifuge and spin at **2,000 x g for 4 minutes;** discard the flow-through from the bottom collection tube and place the filter back into the empty collection tube.
6. Repeat step 5 **two (2) additional times**.
7. Remove the top filter unit from the collection tube and place it into a new Q collection tube (provided)
8. Add 100 µL **Buffer F** to the top of the pink colored filter and incubate for 5 minutes on the bench top.
9. Place the oriented assembly in the centrifuge and spin at **2,000 x g for 4 minutes**.
10. Open the lid of the spin filter and add an additional 50 µL **Buffer F** to the top of the filter and spin the oriented assembly for another **4 minutes at 2,000 x g**. The highly colored conjugate (150 µL) is now located at the bottom of the collection tube. Set the collection tube aside on the bench.

I. Buffer Exchange Conjugate (3 minutes)

1. Prepare a spin column (blue cap) by twisting off the bottom closure and loosening the brown cap (do not remove). Place the spin column into a collection tube (provided).
2. Mark the top of the blue cap using an indelible pen to identify the sample. Also place a vertical mark on the side of each spin column as shown below.



3. Place the assembly into the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor. Use an appropriate balance tube opposite the spin filter
4. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from the collection tube. The column matrix will appear white in color. Place the column back into a **new**, empty collection tube (provided).
5. Remove the blue cap; load 150 µL of the highly colored conjugate to the top of the dry resin bed; loosely recap and place the column back into the collection tube.
6. Orient the spin column mark outward as before and centrifuge at **1,500 x g for 2 minutes**. Use a balance tube opposite the assembly.
7. After centrifugation, transfer the purified conjugate solution (~150 µL) from the bottom of the collection tube to a new 1.5 mL tube and label appropriately.
8. Measure the final protein concentration of the conjugate using a Bradford or BCA protein assay (see Appendix for reference pro

Chapter 4: Appendix

A. Human IgG-R-PE Conjugate (Monoclonal): An example

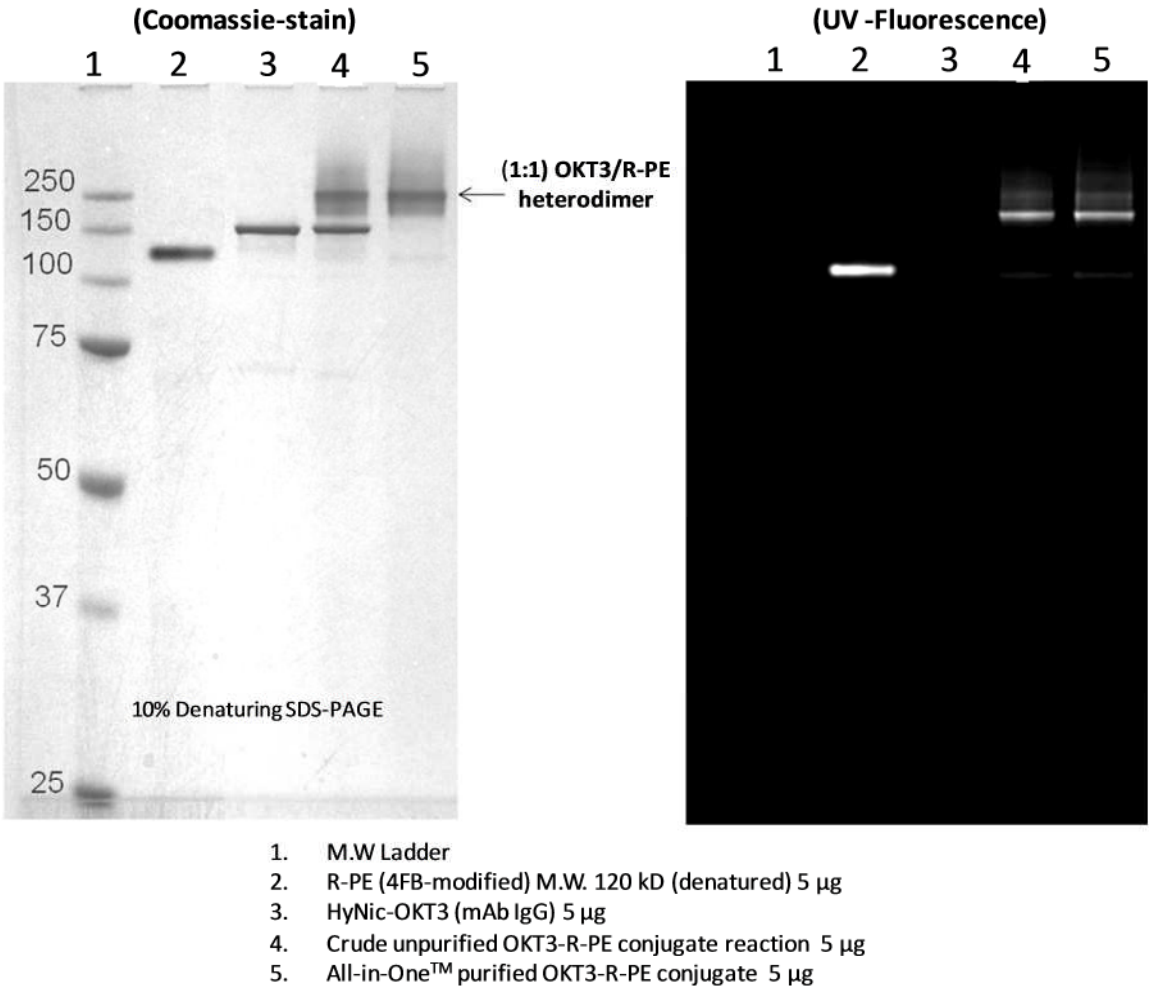


Figure 5. SDS-denaturing PAGE gel images (Coomassie-stained /UV-excitation) of an All-in-One™ R-PE antibody conjugate. Note almost exclusive formation of the desired 1:1 heterodimer (denatured M.W. 270 kD) as well as the purity of the final conjugate (lane 5).

B. Bradford Protein Assay

Solulink highly recommends when IgG is not limiting or its concentration, source, or the quality is unknown that the antibody be assayed for initial protein concentration using a Bradford protein assay prior to using the All-in-One™ conjugation kit. The **quality and quantity** of antibody are critical to the success of the procedure. In this section of the

appendix, we provide a reference protocol for measuring antibody or conjugate protein concentration with Bradford protein assay reagents (not provided).

Bradford Plate Procedure

Required Materials

Bradford Reagent (Bio-Rad, Hercules, CA, Cat. #500-0006)
96-well microtiter plate (standard flat bottom)
PBS (phosphate buffered saline)
P-200 and P-1000 pipettes
Bovine IgG Antibody Standard: 2 mg/ml (Pierce/Thermofisher, Cat. # #23212)
Molecular grade water

Assay Protocol

1. Prepare 2 ml of a Bradford working solution by adding 400 µl dye reagent to 1600 µl molecular grade water (1:4 ratio).

2. Prepare the following protein dilution standards and blank as follows:

   Add 80 µl 2 mg/ml bovine IgG standard to 120 µl PBS (0.8 mg/ml standard)

   Add 75 µl 0.8 mg/ml standard to 25 µl PBS (0.6 mg/ml standard)

   Add 75 µl 0.6 mg/ml standard to 25 µl PBS (0.4 mg/ml standard)

   Add 50 µl 0.4 mg/ml standard to 50 µl PBS (0.2 mg/ml standard)

   Add 50 µl 0.2 mg/ml standard to 50 µl PBS (0.1 mg/ml standard)

   Add 50 µl PBS (buffer blank)

3. Pipette 5 µl of each standard (and blank) along with duplicates of the antibody sample to plate wells.

4. Add 100 µl of previously diluted dye reagent (1:4) to each well and mix thoroughly. Always replace pipette tips between additions.

5. Incubate at room temperature for 5-10 minutes (but no more than 60 minutes).

6. Measure absorbance at 595 nm on a suitable plate reader.

7. A typical Bradford assay result from a commercial plate reader is illustrated in Figure 6 on the next page.

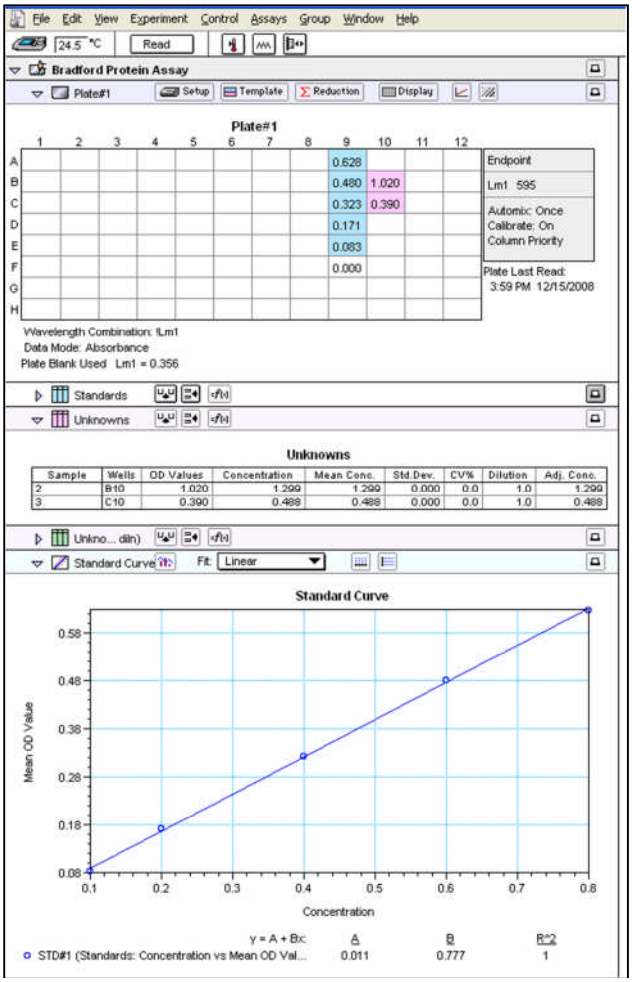


Figure 6. Print out from a Bradford plate-based protein assay.

C. Using a NanoDrop™ to Measure Antibody Concentration

If the antibody sample is free of protein-based carriers (e.g. BSA, gelatin) or certain interfering preservatives such as thimerosal, then a simple non-destructive scan of the IgG sample on a NanoDrop™ spectrophotometer can be used to accurately determine its concentration.

To estimate antibody concentration on a NanoDrop™ spectrophotometer, proceed as follows:

1. Turn on the NanoDrop™ spectrophotometer and click on the NanoDrop™ icon to launch the software.

2. Place a 2 µl drop of molecular grade water on the clean pedestal, click OK.

3. When the main menu appears, select the A_{280} menu option. **Note-** do not use the UV-VIS menu option on the NanoDrop™ to read an antibody sample.

4. After the A_{280} menu appears, **click-off the 340 nm normalization option** using the mouse. **Note-** some NanoDrop™ instruments do not have this feature and therefore can be ignored on these instruments.

5. In the window labeled Sample Type, select 'Other protein E1%' option from the pull-down menu. Enter the appropriate E1% value **(from Table 1)** corresponding to your particular antibody sample type. For example, 14.00 for mouse IgG.

6. Blank the NanoDrop™ spectrophotometer by placing a 2 µl drop of the appropriate sample buffer (e.g. PBS) and click on the 'Blank' icon.

7. Immediately re-click the 'Measure' icon to validate a flat baseline. Clean the pedestal and repeat (if necessary) until a flat baseline is obtained. **Note-**sometimes air bubbles can become trapped on the pedestal during sample loading and cause baseline offsets. If necessary, remove air bubbles and rescan to insure a proper baseline.

8. Transfer 2 µl antibody to the pedestal and click the 'Measure' icon. Wait until the spectrum (220-350 nm) appears in the window. **Note-**for precious or limited samples the 2 µl aliquot can be recovered from the pedestal.

9. Record the antibody concentration directly from the NanoDrop™ display window [mg/ml]. Alternately, calculate the antibody concentration (manually) as illustrated in the following example on the next page.

Example:

A mouse IgG sample in PBS (100 µl @ 1 mg/ml) was scanned as described and its concentration confirmed in the sample below.

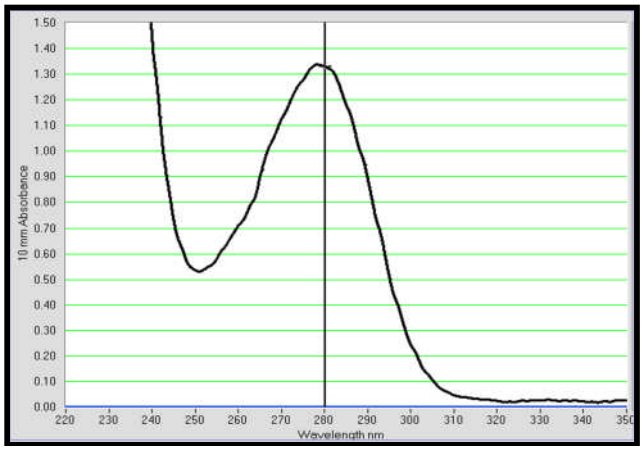


Figure 7. A mouse IgG sample 100 µl @ 1 mg/ml in PBS pH 7.2 was scanned on the NanoDrop™ as described in the text.

Equation: [A_{280} /E1%] x 10 mg/ml = protein concentration (mg/ml)

E1% = mass extinction coefficient (see Table 1)

Mouse IgG @ 1 mg/ml (see Fig. 7)

A_{280} reading (from scan in Figure 6) = 1.34
Antibody E1% value (Table 1) = 14.00

[A_{280} / E1% bovine IgG] x 10 mg/ml = protein concentration (mg/ml)

[1.34 / 14.00] x 10 mg/ml = 0.96 mg/ml

Antibody Source	Antibody E1% (1-cm path)
Human IgG	13.60
Human IgE	15.30
Rabbit IgG	13.50
Donkey IgG	15.00
Horse IgG	15.00
Mouse IgG	14.00
Rat IgG	14.00
Bovine IgG	12.40
Goat IgG	13.60

Table 1. Mass extinction coefficients (E1%) used to determine IgG concentration. E1% = A_{280} of 10 mg/ml solution (1-cm path length).

D. R-PE Absorption Spectrum (Unmodified R-PE)

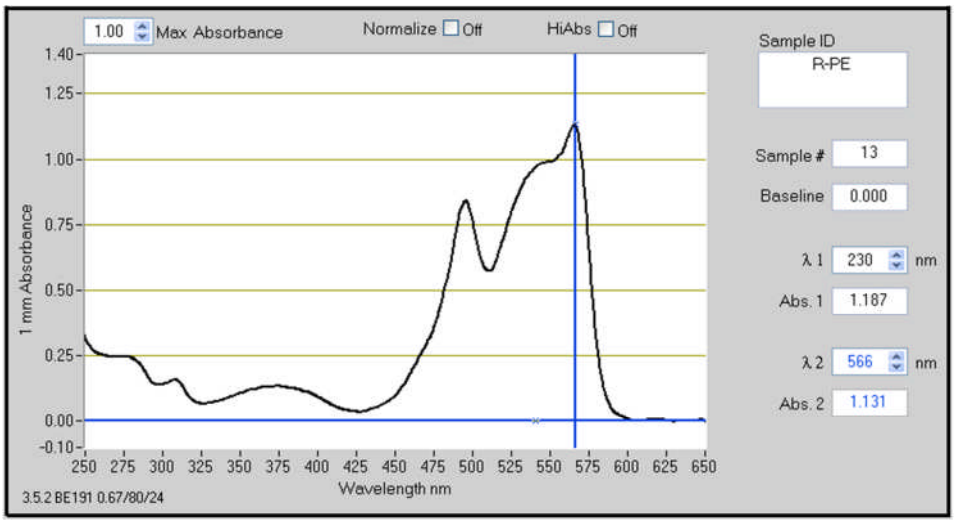


Figure 8. *Absorption spectrum of R-phycoerythrin (250-650 nm) @ 1.38 mg/ml (sodium phosphate buffer, pH 8.0)*

E. R-PE Absorption Spectrum (4FB-modified)

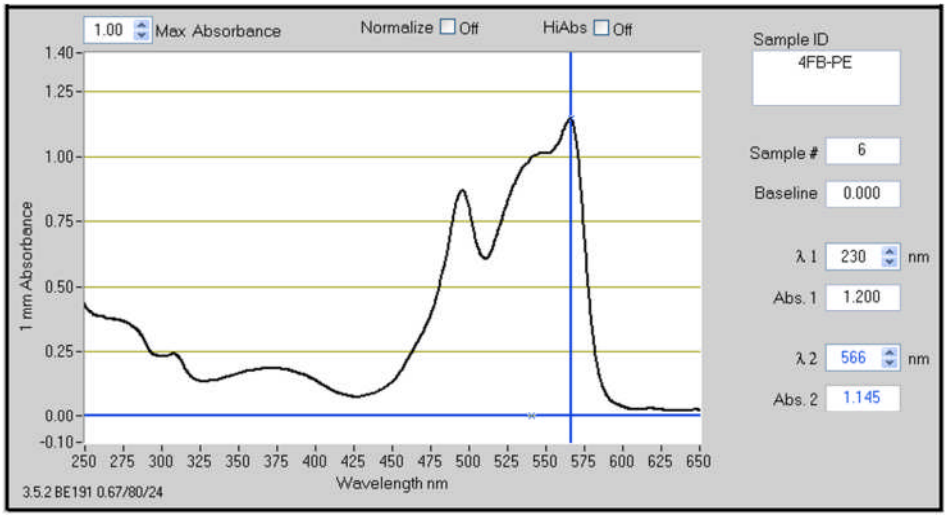


Figure 9. *Absorption spectrum of 4FB modified R-phycoerythrin (250-650 nm) @ 1.38 mg/ml (sodium phosphate buffer, pH 8.0)*

F. Concentration of Dilute Antibody Solutions

The R-PE Antibody All-in-One™ Conjugation protocol requires that initial IgG sample be at a protein concentration of 1 mg/mL and 100 µl. For IgG samples at concentrations significantly less than 1 mg/mL (e.g. 0.1 to 0.5 mg/mL) the IgG must first be concentrated to 1 mg/mL and 100 µL using a suitable ultra-filtration spin filter before proceeding.

Although the All-in-One™ kit does not provide an ultra-filtration spin filter it does provide a recommended reference protocol for concentrating dilute antibody solutions (Figure 10). We routinely employ a VivaSpin 500 (M.W.C.O. 30 kD from Sartorius Stedim, USA) for this purpose. Carefully follow the instructions provided below to avoid irreversible aggregation and loss of IgG on the filter surface. **Note-**dilute antibody solutions may require 125 µg mass of antibody (e.g. 500 ul @ 0.25 mg/ml) since ultra-filtration filters typically recover about 80% of input antibody.

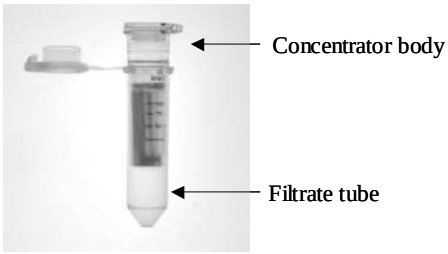


Figure 10. Ultra-filtration spin filter used for concentrating dilute antibody samples prior to the start of All-in-One™ conjugation protocol.

Note- these ultra-filtration spin filters are made to contain and process a maximum volume of 500 µl or less. If a volume greater 0.5 ml is to be concentrated, multiple loadings will be required.

1. Open the lid of a filtration spin filter device.

2. Transfer 500 µl (or less) of dilute protein solution (equivalent to 125 µg antibody) to the center of the filter cup.

3. Close the lid and orient the spin filter in the centrifuge so that the volume markers face toward the center of the centrifuge rotor. Use an appropriate balance tube opposite the spin filter.

4. Centrifuge for **2 minutes @ 5,000 x g**.

   Note -never increase the centrifugation time.

5. Open the filter unit, and pipette the remaining volume up and down gently ~ 20 times to resuspend the antibody back into solution and away from the filter surface.

   Note - take care not to touch or puncture the filter surface during this pipetting step.

6. If necessary, repeat steps 4 and 5 until the volume in the filter cup finally reaches 100 µl (1 mg/ml).

7. Transfer the concentrated IgG solution to a new 1.5 ml microfuge tube and proceed with the conjugation procedure.

G. Troubleshooting Guide

Problem	Possible Cause	Recommended Action
Poor conjugate yield	-initial antibody concentration and volume were incorrect or unknown.	-whenever possible verify the original protein concentration using a Bradford assay to assure 1 mg/ml concentration. -concentrate or dilute the antibody sample to the required range (1 mg/ml and 100 µl) -preservatives can interfere with the accuracy of a Bradford protein assay. Remove all interfering preservatives such as thimerosal or proclin preservative.
Poor HyNic modification	-presence of protein carrier (e.g. BSA or gelatin) is contaminating the antibody sample.	-remove and purify away all protein carriers such as BSA or gelatin using affinity chromatography before proceeding
Poor HyNic modification	-improper mixing of HyNic reaction components -major presences of amine contaminants with the antibody (e.g. 80 mM Tris). -improper storage of S-HyNic reagent can lead to hydrolysis of the NHS ester -initial antibody concentration was too low or too high.	-make sure to properly mix the antibody- HyNic reaction mixture -remove all non-protein amine contaminants such as glycine or Tris before modification -keep and store S-HyNic sealed in the aluminum pouch containing dessicant. measure the initial antibody concentration before proceeding (Bradford or NanoDrop™) -concentrate or dilute the antibody sample into the recommend range (1 mg/ml and 10 µl) before proceeding

Low conjugate and/or antibody recovery	-low spin column recovery volume	-calibrated variable-speed microcentrifuge to the indicated speed and time (e.g. 1500 x g). Lower or higher speeds can adversely compromise protein and volume recovery

H. References

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2. ***Dirksen, A., Hackeng, T., Dawson, P., (2006****). Nucleophilic Catalysis of Oxime Ligations. Angew. Chem. Int. Ed. 45, 7581-7584*

3. ***Dirksen, A., Dirksen, S., Hackeng, T., Dawson, P (2006****). Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. JIAICIS Communications.*

4. ***S. Lim, H. P. Manusu, A. A. Gooley, K. L. Williams and D. B. Rylatt*** *Purification of monoclonal antibodies from ascitic fluid using preparative electrophoresis. Journal of Chromatography A. Vol. 827, Issue 2, 11 December 1998, Pages 329-335.*

5. ***Francesca Chiodi, Dr. Åke Sidén, Eva Ösby (2005).*** *Isoelectric focusing of monoclonal immunoglobulin G, A and M followed by detection with the avidin-biotin system. Electrophoresis, Vol. 6 Issue 3, 124-128.*